INCREASE OF CORTICAL CALCIUM WITH AGE IN THE CELLS OF ELODEA CANADENSIS [1]

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The suggestion has been made frequently that senescence results from progressive accumulation in the cell of materials which either are toxic or obstruct metabolism (Jickeli, 1902; Montgomery, 1906; Child, 1915; Seifritz, 1936; Heilbrunn, 1937). Benedict (1915) formulated the hypothesis that senescence is a result of a decrease in the permeability of cells. Both of these causes of senescence may be the result of an increase with age in the calcium content of the cell membrane, for calcium decreases the permeability of cells to at least some classes of substances (see Heilbrunn, 1937, for literature review). Thus it is conceivable that an increase in the calcium content of the cell cortex could decrease the cellular permeability to its toxic metabolic waste products as well as other substances. A gradual accumulation of these substances in the cell may be a consequence of these preceding changes, and singly or in combination may produce the degenerative changes of senescence. In view of the universality of senescence the author inclines towards the hypothesis that the single or series of related cellular changes sketched above is its universal cause. He proposes therefore to present a series of studies on the possible localized increase of calcium with age in a variety of organisms, and its consequences. It should be noted that Molisch (1938) suggested a relation between the deposition of calcium with age in cell membranes of plants and a decrease in the permeability of cells.

Calcium Changes With Age

Increase of calcium with age is one of the commonest features of the aging process. The calcium content of many human tissues and organs has been shown to be greater in old individuals than in young individuals

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(Burger and Schlomka, 1927; Delbet and Bretau, 1930; Hesse, 1934; Sorsby, Wilcox and Ham, 1935; and Simms and Stolman, 1937).

The work of Novi (1913) on other mammals is in agreement with the findings in human material; he reports that the calcium content of the brain of the guinea pig at birth is very low, but toward the end of the life span it increases markedly. Similarly, Lansing (unpublished observations) found a progressive increase in the calcium content of the rat brain with age. On the other hand, Novi (1913) found that the calcium content of the dog brain is very high in the fetus, is very low in the young animal and finally returns to the high level of the fetus in the old dog. Sorsby, Wilcox and Ham (1935) reported that the amount of calcium in the sclera of the cat eye decreases from birth to adult life, then increases with old age. Lastly, Cahane (1927) concluded that the amount of calcium per gram of fresh muscle of the guinea pig, dog, cat and rabbit decreases with age. It is apparent from Cahane's data, however, that the majority of animals referred to as "old" were actually either immature or young adult animals.

The preceding studies, with the exception of the one by Cahane (1927), demonstrate that the calcium content of mammalian tissues increases after maturity. It is also apparent that the calcium content of fetal tissues may be very high, and that the calcium content decreases in some animals during the period of immaturity.

As far as the writer has been able to determine, there have been no studies of calcium changes with age in plants or invertebrates. The observation that the amount of insoluble salts of calcium increases with age in plants is a common one but has not been put on a quantitative basis. Molisch (1938) points out that calcium oxalate and carbonate are precipitated about the cell walls of plants and increase with age. He suggests the possibility that calcium increases with age in the cell membrane but offers no evidence to support this view. The writer has undertaken a study of calcium changes with age in plants and the invertebrates. This paper deals with the water plant, *Elodea;* a later paper will deal with the calcium changes with age in the invertebrates *Euchlanis,* a rotifer, and *Phagocata,* a planarian.

Mazia and Clark (1936) studying the effects of various stimulating agents on *Elodea* leaves found that upon stimulation calcium is released from the cortex of the cell and combines with the soluble oxalates in the central cell vacuole to form the insoluble crystals of calcium oxalate. The technique of Mazia and Clark suggests a means of determining the amount of calcium in the cortex of *Elodea* cells of varying ages. The following experiments were undertaken to investigate this possibility.

Materials and Methods

Freshly cut stalks of *Elodea canadensis* (*Anachris canadensis*) grown in a glass aquarium at room temperature were employed in all the experiments. The stalks used were a minimum of six inches in length and a maximum of ten inches.

The relative age of an *Elodea* leaf is easy to determine by the position of the leaf on the stalk. Young leaves are located at the apex of the stalk and old leaves are located at the base of the stalk.

The technique used for the study of the calcium content of *Elodea* cells was based upon the observation by Mazia and Clark (1936) that stimulation of the *Elodea* cell results in the combination of the calcium in the cortex of the cell with the oxalates in the vacuole to form insoluble crystals of calcium oxalate. Stimulation was electrical in the present experiments.

Leaves were removed for analysis from various parts of the stalk and were placed on glass slides. Two 1½ volt dry cells were connected in series with an induction coil set for interrupted current. Platinum electrodes were placed directly on the moist leaf and the electrodes were reversed immediately upon the expiration of one half of the total time of stimulation which was two minutes.

Tests were conducted to determine the amount of stimulation required to produce maximal formation of calcium oxalate crystals in the cells. It was found that stimulation for two minutes was adequate to produce the maximal effect in all cases. A young leaf was stimulated for 40 seconds. Thirty-five crystals per cell were formed and subsequent stimulation produced no increase in the number of crystals. Stimulation of an old leaf for 40 seconds produced 15 to 20 crystals, stimulation for 60 seconds produced 30 to 40 crystals, while stimulation for 80 seconds produced 50 to 55 crystals per cell. Further stimulation produced no increase in the number of crystals formed. This strongly suggests that old cells are less susceptible to stimulation than young cells, or rather that they are less irritable; however, no studies were conducted to verify this point.

The description of the calcium oxalate crystals furnished by Mazia and Clark (1936) was used as a basis of identification of the monoclinic and tetragonal crystals found in these experiments. Five small and large tetragonal and large monoclinic crystals, and 46 small monoclinic crystals (in apical and basal leaves) were selected at random for measurement. The lengths and widths were measured by means of a micrometer ocular and the depths by the calibrated fine adjustment screw of the microscope. The depth measurements involve an error of ± 0.5

micron. Small monoclinics average 0.9 ± 0.08 micron in width, 2.5 ± 0.09 micron in length and 1.0 micron in depth. Small tetragonal crystals average 3.3 ± 0.08 micra in width, 3.3 ± 0.05 micra in length and measure 2.0 micra from apex to apex of the crystal. Large tetragonal crystals average 6.8 ± 0.21 micra in width, 7.2 ± 0.00 micra in length and 4.5 micra from apex to apex of the crystal. Large monoclinic crystals average 3.3 ± 0.25 micra in width, 6.2 ± 0.32 micra in length and 3.0 micra in depth.

The volumes of the various types of crystals were computed on the basis of the above measurements. As the monoclinic crystals are rectangular parallelopipeds, the product of the three linear dimensions of the monoclinic crystals was employed for the volume calculation. Since the tetragonal crystal is essentially two pyramids whose bases are in juxtaposition the formula for computing its volume is two times the area of the base times the height of the pyramid divided by three, i.e., twice the volume of a pyramid. Thus the average volume of the small monoclinic crystals is 2.2 ± 0.33 cubic micra, small tetragonal is 21.5 ± 0.7 cubic micra, large tetragonal is 222.3 ± 13.4 cubic micra and large tetragonal crystal is 62.6 ± 7.0 cubic micra. The calcium content of the cells studied was determined by making counts of the number of crystals present after stimulation and computing the total volume of crystalline material.

Since the amount of crystalline material formed in these experiments could possibly be correlated with the amount of cell surface present as well as the concentration of calcium in the cell cortex, it was necessary to determine the total amount of surface for the various cells.

Measurements with a micrometer ocular were made of six cells in the tip region of young and of old leaves. The sum of the areas of the surfaces of the cell was used in the computation of the total cell surface. The total surface of the mean apical cell in apical leaves was 2919 square micra and of the mean apical cell of basal leaves was 3139 square micra. Thus, the total cell surface of apical cells of basal leaves was only seven per cent greater than that of apical cells of apical leaves.

Quantitative determinations of the oxalate content of apical regions of young leaves were made. For each determination 75 leaves were used; the anterior fifth of each leaf was cut off and put in a porcelain crucible containing distilled water. The material was thoroughly crushed and the supernatant fluid decanted. The residue was washed several times with distilled water and the washings added to the material previously removed. The remaining pulp was filtered and washed, the filtrate again being added to the previous wash solutions. The solution was rendered alkaline with NH_4OH and 2 cc. N/3 $CaCl_2$ was added in

order to obtain complete precipitation of the oxalate. The solution was then filtered and the residue dissolved in 15 cc. of hot H_2SO_4 (1:5). The filter paper was washed with an additional 15 cc. of H_2SO_4 at 70° C., and the filtrate was added to the solution previously obtained. The solution was kept at 70° C. and titrated against 0.01 N $KMnO_4$. Two experiments on tip regions gave the following results: 0.0176 and 0.0107 gm. oxalic acid per gram of leaf material.

Results

The cells in *Elodea* leaves are arranged in longitudinal rows which may be readily traced along the length of the leaf. In five series of experiments counts were made of the number of crystals formed after stimulation in the tip regions of leaves at different positions on the stalk. Only the upper surface of the leaf was studied, and rows of cells ap-

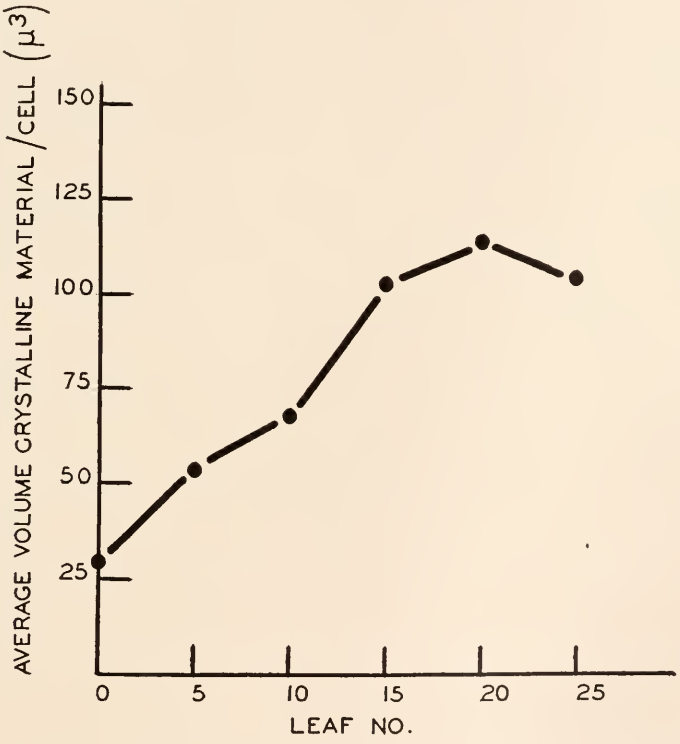

Fig. 1. Graph showing the average volume of crystalline material per cell formed after stimulation of *Elodea* leaves of different ages. The tip leaves are represented by 0, basal leaves by 25.

proximately halfway between the midrib and the edge of the leaf were selected for analysis. Beginning at the apex of the stalk every fifth leaf was removed and stimulated. Only the first 20 cells from the apex of the leaf were used and every fourth cell was counted so that for each leaf there were five cells counted. The computed data for the total amount of crystalline material in these experiments are shown graphically in Figure 1. Using the volume data mentioned previously it was determined that the various leaves contained the following amounts of crystalline material:

Apical leaf	29.5	cubic micra
Fifth leaf	55.7	" "
Tenth leaf	67.8	" "
Fifteenth leaf	102.3	" "
Twentieth leaf	114.0	" "
Twenty-fifth leaf	104.3	" "

In contrast with the group of apical leaves studied in which no tetragonal crystals were found, one large tetragonal crystal was noted in the fifth leaf group, one small tetragonal crystal in the tenth and fifteenth leaf groups, one large and five small tetragonals in the twentieth leaf group, and seven small tetragonal crystals in the twenty-fifth leaf group.

Discussion

Mazia and Clark (1936) state that stimulation of *Elodea* cells causes a release of calcium from the cortex into the cell interior where the free calcium combines with the soluble oxalates in the central cell vacuole to form the crystals of calcium oxalate.

The experiments reported by the writer show clearly that there is an increase with age in the amount of calcium oxalate formed after stimulation of the cells of *Elodea,* and it may be concluded that the cell cortex of old *Elodea* cells contains more calcium than young *Elodea* cells. The quantitative analyses of the oxalate content of young leaves conducted by the writer (see section on methods) show that oxalates are present as at least 1 per cent of the total wet weight of leaf material. As this concentration of oxalate is far in excess of the concentration of calcium in living tissues, an increase with age in the amount of oxalates would have no effect on the amount of calcium oxalate formed after stimulation.

The question then arises whether there is actually an increase with age in the amount of calcium per unit of cell surface or whether there is an increase in cell surface which would account for the additional calcium in old cells. The cell measurements referred to earlier show a

7 per cent difference in cell surface between apical cells from tip and basal leaves. Obviously, a 7 per cent increase in cell surface is not adequate to account for the 300 per cent increase in the amount of crystalline material observed in the present experiments, and it may be concluded that the concentration of calcium per unit of cell surface increases with age in the cortex of *Elodea* leaf cells.

The results obtained in these experiments demonstrate that calcium increases with age in plants as well as mammals. The amount of cortical calcium per unit of plant cell surface increases with age.

Summary

By means of electrical stimulation of the leaves of *Elodea canadensis,* insoluble crystals of calcium oxalate were formed in the central vacuole of cells of different ages. There was an increase with age in the amount of crystalline material formed; moreover, the amount of cortical calcium per unit of cell surface increased with age.

LITERATURE CITED

Benedict, H. M., 1915. Senile changes in the leaves of Vitis vulpina L., and certain other plants. *Cornell Agr. Exp. Sta. Mem.,* **7**: 273–370.

Burger, M., and G. Schlomka, 1927. Beitrage zur physiologischen Chemie des Alterns der Gewebe. *Zeit. Exp. Med.,* **55**: 287–302.

Cahane, M., 1927. Teneur dur tissu musculaire et du sang en calcium magnesium et potassium au point de vue ilikibiologique. *C. R. Soc. Biol.,* **96**: 1168–1169.

Child, C. M., 1915. Senescence and Rejuvenescence. Chicago.

Delbet, P., and P. Bretau, 1930. Vieillissement et magnesium. *Bull. Acad. Med. Paris,* 3s., **103**: 256–266.

Heilbrunn, L. V., 1937. An Outline of General Physiology. Philadelphia.

Hesse, M., 1934. Über das wesen der hyalinose der kleinen arterien auf grund der untersuchungen von kindermilzen. *Virch. Arch. f. Path. Anat. u. Physiol.,* **292**: 465–478.

Jickeli, C. F., 1902. Die Unvollkommenheit des Stoffwechsels. Berlin.

Mazia, D., and J. Clark, 1936. Free calcium in the action of stimulating agents on Elodea cells. *Biol. Bull.,* **71**: 306–323.

Molisch, H., 1938. The Longevity of Plants. English translation. New York.

Montgomery, T. H., 1906. On reproduction, animal life cycles, and the biological unit. *Trans. Texas Acad. Sci.,* **9**: 75.

Novi, I., 1913. Le calcium et le magnésium du cerveau dans les différents âges. *Arch. Ital. Biol.,* **58**: 333–336.

Seifritz, W., 1936. Protoplasm. New York.

Simms, H. S., and A. Stolman, 1937. Changes in human tissue electrolytes in senescence. *Science,* **86**: 269–270.

Sorsby, A., K. Wilcox, and D. Ham, 1935. Calcium content of the sclerotic and its variations with age. *Brit. Jour. Ophthal.,* **19**: 327–337.